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An Examination of the Utility of Photogenerated Reagents by Using α-Chymotrypsin

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As a test of the labelling characteristics of photogenerated reagents, an aryl azide was photolysed in the aromatic-binding locus of a protein of known tertiary structure. The acyl-enzyme derived from the reaction of α-chymotrypsin with the *p*-nitrophenyl ester of *p*-azido[^{14}C]cinnamate was isolated and photolysed. About 60% of the acyl group is covalently bound to the protein after photolysis and deacylation, and labelled enzyme is inactive. The covalently attached label is localized in the C chain of chymotrypsin, and there are firm indications that the major labelled tryptic fragment of the C chain is that which constitutes the aromatic-binding locus of the enzyme. The high degree of labelling of that portion of the protein molecule predicted on the basis of the known chemistry and structure of α-chymotrypsin, provides gratifying confirmation of the utility of the photolabelling method.

Since the original experiments of Westheimer and his group (Singh *et al.*, 1962; Shafer *et al.*, 1966) the use of photogenerated reagents for the investigation of ligand-binding sites has received considerable attention (see Knowles, 1972, and references therein). A number of proteases (Singh *et al.*, 1962; Shafer *et al.*, 1966; Vaughan & Westheimer, 1969; Hexter & Westheimer, 1971*a*,*b*; Stefanovsky & Westheimer, 1973), dehydrogenases (Chaimovich *et al.*, 1968; Browne *et al.*, 1971; White & Yielding, 1973) and other enzymes (Cooperman & Brunswick, 1973; Martyr & Benisek, 1973) have been labelled, and other receptor sites are under investigation (Brunswick & Cooperman, 1971, 1973; Converse & Richards, 1969; Yoshioka *et al.*, 1973; Katzenellenbogen *et al.*, 1973; Winter & Goldstein, 1972). Labelled amino acids have been obtained in some of these studies, although only in one case, involving the labelling of an antibody to the photoprecursor of a nitrene reagent (Fleet *et al.*, 1972), have labelled peptides been isolated and located in the primary sequence of the receptor protein. Thus although the approach continues to be explored at the level of the development of new reagents (Smith & Knowles, 1973) and the investigation of more complex biological systems (Waser *et al.*, 1970; Kiefer *et al.*, 1970; Guthrow *et al.*, 1973; Hanstein, 1973; Das Gupta & Rieske, 1973), there is a dearth of information about the labelling of systems whose three-dimensional structure is known, against which to test our predictions about labelling patterns.

To examine therefore the labelling characteristics of photogenerated reagents, it is necessary to subject a system of known tertiary structure to such a reagent. Our earlier study (Fleet *et al.*, 1972) with the 4-azido-2-nitrophenyl group caused us to opt for a protein that could be labelled by an aryl azide in a way that could be predicted on the basis of established protein chemistry and tertiary structure. The system of choice is α-chymotrypsin. This enzyme is extremely well characterized in both structural and mechanistic terms. The primary substrate-binding locus is a hydrophobic cleft [the 'tosyl hole' (Steitz *et al.*, 1969)] in which the large aromatic side chains of amino acid substrates of the enzyme are bound. This binding site is well characterized, both in terms of its effect on the kinetics of substrate reaction (Knowles, 1965; Ingles & Knowles, 1967) and in terms of its three-dimensional structure (Blow & Steitz, 1970). Further, the mechanistic course of α-chymotrypsin-catalysed reactions is well charted, and the molecular structure of the intermediate acyl-enzyme can be predicted in some detail (Blow & Steitz, 1970). This enzyme is therefore ideal for the proposed investigation. There is a unique binding site, whose three-dimensional architecture is known, which interacts strongly with aryl derivatives. Moreover, if an appropriately stable aromatic acyl-enzyme can be synthesized, then the aryl group will be held covalently in the binding locus of the protein. If the aryl group is an aryl azide, then the effectiveness of protein photolabelling by an aryl nitrene can be investigated.

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Experimental

Materials

Sodium [2-^{14}C]malonate (12mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). α-Chymotrypsin (from bovine pancreas) and trypsin were obtained from Miles-Seravac (Pty.) Ltd. (Maidenhead, Berks., U.K.). DEAE-cellulose (DE52) was obtained from Reeve-Angel Scientific Ltd. (New Bridge Street, London EC4V 6AY, U.K.). Sephadex G-25 and G-50 was obtained from Pharmacia (G.B.) Ltd. (Uxbridge, London W5 5SS, U.K.).

Aqueous solutions of 8M-urea were subjected to chromatography on Amberlite MB-3 ion-exchange resin and had a specific conductivity of less than 10μmho. Dimethylformamide was purified by distillation under reduced pressure from P_2O_5. Buffer solutions were made up with deionized water, and all other reagents were of the highest grade available. *p*-Aminobenzaldehyde was prepared from *p*-nitrotoluene by the method of Campaigne *et al.* (1963).

p-Azidobenzaldehyde. *p*-Aminobenzaldehyde (15g) was added to a mixture of ice (50g) and 12M-HCl (65ml), and the resulting solution was cooled to −10°C. A solution of $NaNO_2$ (10g) in water (50ml) was added slowly with stirring over 30min, at the end of which the excess of nitrite was destroyed by the addition of urea. The mixture was filtered, and to the filtrate a solution of NaN_3 (9g) in water (50ml) was added slowly. The resulting solution was extracted with ether (3×50ml), and the combined ethereal extracts were washed with 2M-HCl (100ml) and then with water (100ml). The solution was dried over anhydrous $MgSO_4$, after which the ether was removed under reduced pressure. The resulting oil was subjected to chromatography on silica gel by using light petroleum (b.p. 30–40°C)–ether (19:1, v/v) as the eluent. The solvent was removed under reduced pressure, leaving a light-yellow oil (11g). (Found: C, 56.9; H, 3.5; N, 28.4; $C_7H_5N_3O$ requires: C, 57.2; H, 3.4; N, 28.3%). The proton n.m.r. spectrum (at 100MHz) in [^{2}H]chloroform solution showed peaks at 0.1τ (singlet, 1 proton), 2.18τ [doublet (*J*9Hz), 2 protons], 2.92τ [doublet (*J*9Hz), two protons]; $\lambda_{max.}$ 288nm (ε19000); $\nu_{max.}$ 2100, 1685, 1595 and 835cm^{-1}.

p-Azidocinnamic acid. Malonic acid (198mg) and *p*-azidobenzaldehyde (298mg) were dissolved in dry pyridine (5ml) containing piperidine (0.1ml). The solution was heated to 100°C for 75min, and then poured on to ice-cold 6M-HCl (10ml) and the pH adjusted to approx. 1. The resulting precipitate was isolated by filtration, dissolved in 1M-NaOH, and the solution boiled briefly with animal charcoal. After filtration, the *p*-azidocinnamic acid was precipitated with 2M-HCl, and extracted into ethyl acetate (2×25ml). The combined ethyl acetate extract was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The remaining solid was recrystallized from ethyl acetate as light-yellow needles, m.p. 192–194°C (decomp.) (yield: 304mg, 85%) (Found: C, 57.3; H, 3.7; N, 21.8; $C_9H_7N_3O_2$ requires: C, 57.2; H, 3.7; N, 22.2%). The proton n.m.r. spectrum (at 100MHz) in [^{2}H]chloroform solution showed peaks at 2.28τ [doublet (*J*8Hz), 2 protons], 2.35τ [doublet (*J*16Hz), 1 proton], 2.87τ [doublet (*J*8Hz), 2 protons], 3.53τ [doublet (*J*16Hz), 1 proton]; $\lambda_{max.}$ 303nm (ε22000); $\nu_{max.}$ 2100, 1685, 1595, 1290, 1220, 980 and 820cm^{-1}.

p-Nitrophenyl p-azidocinnamate. *p*-Azidocinnamic acid (189mg), *p*-nitrophenol (139mg) and dicyclohexylcarbodi-imide (203mg) were dissolved in dichloromethane (30ml) at 0°C. After 1h the solution was allowed to reach room temperature, and the mixture stirred for 72h. The mixture was then filtered, and the solvent was removed from the filtrate under reduced pressure. The residue was then triturated with ethyl acetate (2×10ml). After removal of the ethyl acetate, the residue was recrystallized from ethanol as light-yellow needles, m.p. 142–144°C (yield: 240mg, 77%) (Found: C, 58.0; H, 3.6; N, 17.7; $C_{15}H_{10}N_4O_4$ requires: C, 58.1; H, 3.2; N, 18.1%). The proton n.m.r. spectrum (at 100MHz) in [^{2}H]chloroform solution showed peaks at 1.58τ [doublet (*J*9Hz), 2 protons], 2.01τ [doublet (*J*16Hz), 1 proton], 2.29τ [doublet (*J*9Hz), 2 protons], 2.51τ [doublet (*J*9Hz), 2 protons], 2.80τ [doublet (*J*9Hz), 2 protons], 3.31τ [doublet (*J*16Hz), 1 proton]; $\lambda_{max.}$ 325nm (ε35000); $\nu_{max.}$ 2130, 1730, 1630, 1598, 1350, 990cm^{-1}. A molecular ion of *m/e* 310 was observed in the mass spectrum.

p-Nitrophenyl p-azido[^{14}*C*]*cinnamate.* Sodium [2-^{14}C]malonate (200μCi) was diluted with malonic acid (41mg) and dissolved in pyridine (2ml), with *p*-azidobenzaldehyde (102mg) and piperidine (0.1ml). The solution was heated at 100°C for 1h, and then poured into cold 6M-HCl (5ml) and the pH adjusted to 1. The solution was filtered, and the residue dissolved in aqueous 2.5M-NaOH (5ml). This solution was decolourized with animal charcoal, and after acidification the cinnamic acid was extracted into ethyl acetate (2×10ml). The solvent was removed under reduced pressure and *p*-nitrophenol (70mg) in dichloromethane (10ml) was added. The solution was cooled to 0°C and dicyclohexylcarbodi-imide (100mg) was added. The mixture was stirred at 0°C for 3 days, after which the solvent was removed under reduced pressure and the residue triturated with ethyl acetate (2×10ml). After removal of the solvent, the *p*-nitrophenyl ester was recrystallized from ethanol as light-yellow needles, m.p. 142–143°C (yield: 70mg, 55%). The specific radioactivity was 8.65×10^{5}d.p.m./μmol. It was checked for radiochemical purity by elution on silica t.l.c. plates with benzene. Scintillation counting of strips from the t.l.c. plate showed only one radio-

active peak corresponding to the single spot of the ester.

Methods

U.v. spectra were measured with a Pye Unicam 1800 instrument. I.r. spectra were recorded with a Perkin Elmer 257 spectrophotometer. Mass spectra were determined by Dr. R. Aplin in this Department with an A.E.I. MS9 instrument. Proton n.m.r. spectra were recorded by Mrs. E. Richards in this Department with a Perkin Elmer R14 instrument at 100MHz. Scintillation counting was done with a Beckman LS100 liquid-scintillation spectrophotometer by using a toluene–ethanol mixture (Knowles *et al.*, 1969). Melting points were determined on a Kofler block and are uncorrected. Micro-analyses were performed by Dr. Strauss in this Department. Amino acid analyses were done by Miss J. Sykora by using a Jeol JLC 5AH instrument. Samples were hydrolysed *in vacuo* in 6M-HCl at 110°C for 20h, in the presence of 1% (w/v) phenol. Photolysis experiments were done in glass vessels, irradiated by two Mazda 125W MB/V pearl-glass lamps at 0°C in a bath containing 0.072M-$NaNO_2$. The distance between sample and the light sources was approx. 10cm.

Results and Discussion

Acylation of α-chymotrypsin by p-nitrophenyl p-azidocinnamate

Kinetic experiments were performed to confirm that *p*-nitrophenyl *p*-azidocinnamate was a substrate for α-chymotrypsin, and to define conditions under which the acyl-enzyme intermediate might best be formed. The limited solubility of the ester in water led to the use of aqueous dimethylformamide (20%, v/v). In this medium, α-chymotrypsin is still active (Elödi, 1961) and the ester was added to the enzyme as a concentrated solution in acetone. The hydrolysis of the substrate was monitored by the increase in E_{404} owing to the *p*-nitrophenol anion, and at pH6.8 and 7.6 showed a rapid initial burst followed by the slower steady-state turnover of the enzyme. The initial burst was complete within 5s, and extrapolation of the steady-state reaction showed that approx. 80% of the enzyme had been acylated on a weight basis. This amounts to all the available active sites as assayed by the chymotrypsin titrant, the iodide salt of *p*-trimethylammonium *p*-nitrophenylcinnamate (Knowles & Preston, 1968). It therefore appears that the reagent *p*-nitrophenyl *p*-azidocinnamate is a well-behaved substrate for α-chymotrypsin. For the isolation of acyl-chymotrypsins, a low pH is usually used, and below pH6 the acylation was followed from the difference spectrum at 340nm. Exploratory experiments showed that acylation of the enzyme was essentially complete within 2min at pH5, and that the turnover subsequent to this was very slow, allowing the pH to be lowered to 3 after the acylation reaction was over.

Despite the low solubility of the ester, and because of the established ability of chymotrypsin to solubilize its substrates, the conditions chosen for the preparation of reasonable quantities of acyl-enzyme were: *p*-nitrophenyl *p*-azidocinnamate (0.25mM) and α-chymotrypsin (0.1mM) in 0.1M-sodium citrate buffer, pH5.0, 20% (v/v) dimethylformamide, 2.5% (v/v) acetone. After 30min the pH was lowered to 3 by using 2M-HCl, and the acyl-enzyme was isolated by gel filtration on Sephadex G-25. When the concentration of acyl-enzyme was assayed either by scintillation counting (by using ^{14}C-labelled ester) or by titration of the remaining active enzyme (Knowles & Preston, 1968), it was clear that 80% of the active enzyme had been acylated.

To ensure that deacylation could be effected, and to check that no photolabelling had occurred up to this stage, the pH of the acyl-enzyme solution was adjusted to 7 and hydroxylamine was added (to 0.2M). After 6h at room temperature, gel filtration on Sephadex G-25 demonstrated that less than 1% of the radioactivity was now associated with the protein. It was found important to exclude light rigorously during all stages of the acyl-enzyme preparation if a clean deacylation was to be achieved.

Photolysis of p-azidocinnamoyl-α-chymotrypsin

Several small-scale photolyses of the isolated acyl-enzyme were performed at 0°C and pH3 (see under 'Methods'). After 8–12h photolysis the acyl-enzyme was subjected to the deacylation treatment described above, on the reasonable basis that hydroxylamine affects deacylation of the photolysed acyl-enzyme, and it was found that about 60% of the radioactivity was still bound to protein. Active-site titration of this material demonstrated that the amount of active enzyme remaining plus the labelled enzyme (presumed to be inactive) was close to the original amount of enzyme present. This strongly suggests that only labelled enzyme has been inactivated, and that the label is, as expected, at the active site. It should be mentioned that the yield of labelled protein obtained here is, by the standards of other photolabelling experiments, very high. We expect, of course, that photolysis will generate the aryl nitrene at the bottom of the 'tosyl hole' of chymotrypsin, where wasteful reaction of nitrene with solvent is unlikely, and where migration of the label out of the site is impossible without hydrolysis of the serine ester link of the acyl-enzyme. It is nevertheless a gratifying vindication of the method that, in such a situation of tight binding to a protein-receptor site, covalent labelling by more than 60% of the bound ligand has been achieved on photolysis.

In a large-scale preparation, 500mg of enzyme in buffer (200ml) was acylated with ^{14}C-labelled ester

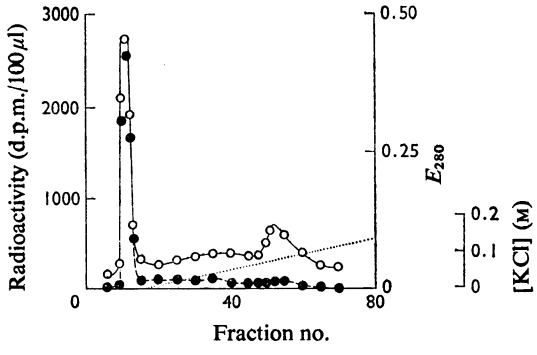

Fig. 1. *Separation of the B and C chains of labelled α-chymotrypsin after performic acid oxidation*

After performic acid oxidation of the labelled protein (for details, see the text), the mixture was subjected to gel filtration on Sephadex G-25. The protein pool was then subjected to ion-exchange chromatography on a column (32cm×2.7cm) of DEAE-cellulose equilibrated with 0.1M-sodium citrate buffer, pH5.0, 4M in urea. Elution was achieved by using a linear salt gradient (2 litres), 0–0.2M-KCl. Fractions of volume 25ml were collected. ○, E_{280}; ●, radioactivity (d.p.m./100μl). C-chain pool, fractions 9–15; B-chain pool, fractions 50–56.

Table 1. *Amino acid analysis (short-column results only) of isolated B and C chains of labelled α-chymotrypsin after performic acid oxidation*

For details see the text.

	B-chain pool		C-chain pool	
	Expected	Found*	Expected	Found*
Lysine	8	7.55	6	6.50
Histidine	2	2.10	0	0.22
Arginine	1	1.04	2	1.87

* On the basis of alanine = 11 (B chain) and alanine = 10 (C chain).

(16mg) to yield 77% acylated enzyme (5.6×10^5 d.p.m./μmol). After photolysis and deacylation with hydroxylamine, the labelled protein was dialysed exhaustively against 10mM-citric acid followed by deionized water, and then freeze-dried. Amino acid analysis showed that the pool contained 9.6μmol of protein with specific radioactivity of 3.7×10^5d.p.m./μmol, corresponding to 43% total labelling of the enzyme.

Localization of the radioactive label

α-Chymotrypsin contains three polypeptide chains and five disulphide bridges. The A chain has 13 residues, the B chain has 131, and the C chain 97 (Hartley *et al.*, 1965). Although the chains can be separated readily, the B and C chains have very low solubility, and Hartley (1964) had to use 8M-urea and 2-carboxymethylation to effect a column separation. Even so tryptic digestion of either chain results in substantial amounts of insoluble 'core', that from the C chain being insoluble even in 8M-urea or 30% acetic acid (Hartley, 1964). This problem was overcome in the present work by using the performic acid-oxidized chains. Thus the photolabelled enzyme was dissolved in formic acid–methanol (5:1, v/v) and oxidized with performic acid for 6h at −5°C. A fresh portion of performic acid was added after 2.5h. The mixture was subjected to gel filtration on Sephadex G-25 in sodium acetate buffer, pH5.0, 4M in urea. The pooled protein fractions (containing the B and C chains) were then chromatographed on DEAE-cellulose. As shown in Fig. 1, the B chain and C chain were well-separated. Amino acid analysis (see Table 1) confirmed the identity of the chains.

From Fig. 1 it is clear that the radioactivity is predominantly associated with the C chain. The specific radioactivity of the B chain was 6.0×10^4d.p.m./μmol, corresponding to 6.9% labelling, and of the C chain, 2.75×10^5d.p.m./μmol, corresponding to 32% labelling. From the three-dimensional structure of α-chymotrypsin, we know that the aromatic-binding locus (the 'tosyl hole'), which results in the binding and catalytic specificity of the enzyme, is made up entirely of residues derived from the C chain. This contrasts with the general area of the active site, which is made up of amino acid residues from both the B and the C chains. The highly preferential labelling of C-chain residues therefore confirms the view that the photolabile reagent is indeed constrained within the 'tosyl hole', and that on photolysis, reaction at this locus dominates the labelling pattern.

As the performic acid-oxidized C chain is soluble, some effort was made further to localize the radioactive label within the chain. On proteolysis of the C chain with thermolysin, trypsin, chymotrypsin or pepsin, soluble digests were obtained that resulted on vertical-tank paper electrophoresis in well-resolved peptide 'maps'. The radioactivity was, however, spread over much of the digests, which suggests that there are several different labelled peptides. After large-scale tryptic digestion of labelled C chain, gel filtration on Sephadex G-50 resulted in the profile shown in Fig. 2. Eight tryptic fragments are expected, containing 27, 25, 15, 15, 6, 6, 2 and 1 amino acids, of which those absorbing at 280nm will contain 27, 15 and 6 amino acid residues. The elution volumes of the three major absorbing peaks in Fig. 2 are in reasonable correspondence with those predicted for peptides of this length, and it is evident that the two peaks of radioactivity correspond to oligopeptides containing about 25–30 and 15–25 residues respectively. The larger radioactive peak can be tentatively

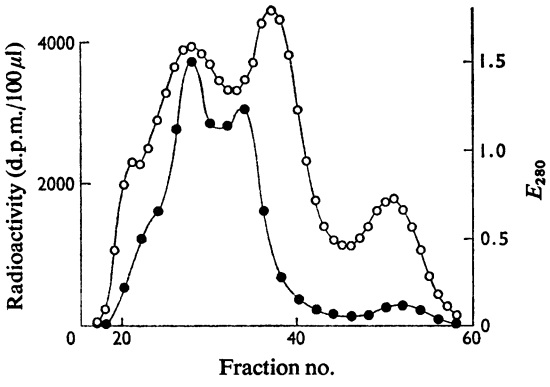

Fig. 2. *Elution profile for tryptic digest of the labelled C chain of α-chymotrypsin*

Labelled C chain (1.08×10^6 d.p.m.) was dissolved in 0.5% (w/v) NH_4HCO_3 (12ml) and digested with trypsin (0.4mg) for 6h at 38°C. The solution was then freeze-dried; the residue was dissolved in 0.5% (w/v) NH_4HCO_3 and subjected to gel filtration on a column (150cm × 1cm) of Sephadex G-50 (fine) eluted with the same solution. Fractions of volume 2.5ml were collected. ○, E_{280}; ●, radioactivity (d.p.m. in 100μl).

identified with the 27-membered fragment from asparagine-204 to arginine-230 (Hartley, 1974). This region of the sequence of the C chain constitutes the aromatic binding locus of α-chymotrypsin, which is made up of valine-213, serine-214, tryptophan-215, glycine-216, serine-217, cysteine-220, serine-221, glycine-226, serine-189 and serine-190. It appears, therefore, that the expected portion of the C chain contains the bulk of the radioactive label.

Attempts were made further to delineate the sites of attack of the photogenerated nitrene, but on fractionation of the oligopeptides from Fig. 2 by electrophoresis or ion-exchange chromatography, increasing numbers of closely similar peptides were obtained. This is not surprising, since any extremely reactive reagent will react randomly with available bonds nearby, and result in a number of related products differing only slightly from one another in structure.

In summary, α-chymotrypsin was covalently labelled with a specific aryl nitrene reagent. A high extent of labelling was achieved, and it was shown that the bulk of the label is located in the aromatic-binding locus of the enzyme, as predicted from the known specificity, mechanism and tertiary structure of the protein. From the results of this study, the photoaffinity-labelling method rests on firmer ground.

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